Optical Recording of Intracellular pH in Respiratory Chemoreceptors

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We studied the spontaneously active *in vitro* tadpole brainstem and recorded whole nerve respiratory activity while simultaneously visualizing intracellular pH (pHi) dynamics using the pH-sensitive dye, 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF, AM). The isolated, superfused tadpole brainstem is well oxygenated and retains synaptic connectivity among respiratory central pattern generators, central respiratory chemoreceptors, and respiratory motor neurons. We generated a calibration curve to correlate the emitted fluorescence of BCECF to pHi. In addition, we demonstrated that the dye loading protocol that we established labeled an adequate number of cells and did not disrupt spontaneous respiratory rhythmogenesis or the respiratory response to central chemoreceptor stimulation. Validation of the use of the pH sensitive dye BCECF in this preparation will permit further characterization of the pH regulatory responses of central respiratory chemoreceptors and allow correlation between the changes in pHi in central chemoreceptors and respiratory motor output recorded from cranial nerves. (*Ethn Dis.* 2010;20[Suppl 1]:S1-33–S1-38)

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Introduction

The characterization of the cellular mechanisms involved in CO_2/H^+ chemoreception has been the focus of recent research.[1,2] Unequivocally identifying a chemosensitive cell as being respiratory in nature continues to be problematic when studying central respiratory chemoreception. It is difficult to establish firm causal relationships between stimulus, effector elements and responses in intact preparations because each neuron makes only a small contribution to the overall response – different patterns of neuronal activity can only be correlated with particular responses. In reduced preparations, causal links may be established between sequential neural elements, but the biological importance of any particular response is moot since the neural activities are frequently unconnected to meaningful physiological responses. Brain areas involved in respiratory chemosensitivity have been identified by the discrete application of CO_2/H^+ and differences in rates of intracellular pH (pHi) regulation between putative chemosensitive and non-chemosensitive cells have been proposed.[3–5] In addition, separate studies using pH-sensitive dyes and whole cell recordings have found significant correlations between pHi and neuronal firing rate.[5] While these studies provide valuable information regarding the effects of CO_2/H^+ on cellular mechanisms, it has not been possible to attribute changes in respiratory motor output to the quantifiable changes in CO_2 or pH in any particular nucleus or cell population. Addressing the relationship between measurable changes in intracellular pH and respiratory motor output requires simultaneous measurements of pHi dynamics and whole nerve respiratory output in a single preparation. The application of the *in vitro* tadpole brainstem preparation for simultaneous measurements of pHi using the pH-sensitive dye BCECF provides a novel model to determine if there is a quantifiable relationship between changes in pHi in specific cells and neural activity related to ventilation.

The pH Sensitive Dye BCECF

Fluorescein diacetate derivatives have been previously used to determine the intactness of cellular membranes, the presence of intracellular esterases, intracellular pH, and more recently to elucidate mechanisms of pHi regulation.[6–9] The dye, 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein - acetoxymethyl ester (BCECF-AM) is a pH sensitive analog of fluorescein. It is synthesized from carboxyfluorescein by adding two extra carboxylate groups via short alkyl chains and has a pKa of 6.97 making it ideal for reporting pHi at physiological conditions.[10] The addition of the acetoxymethyl (-AM) makes BCECF membrane permeable and so the dye passively enters all cells.[11] Once inside the cell, hydrolysis of the acetyl ester linkage by enzymatic cleavage regenerates the less permeable and fluorescent original compound.[6] BCECF is a dual excitation ratiometric dye that when excited at 440 nm (its isosbestic point) is pH insensitive and when excited at 495 nm is pH-sensitive.[12] BCECF fluorescence reflects only cytoplasmic changes in pH and allows rapid kinetic changes of pH as small as 0.01 to be monitored.[13]

To determine if the use of the pH sensitive dye BCECF can be incorporated to study pHi changes in the *in*

vitro tadpole brainstem preparation, several important criteria must be established. First, we must determine if cells on the ventral surface of the tadpole brainstem contained the intracellular esterases necessary for cleavage of the –AM portion of the dye making it membrane impermeable. Second, we must develop a dye loading protocol that will allow us to load and visualize an adequate number of brainstem neurons. Finally, since hydrolysis of the acetyl ester linkage is followed by the spontaneous release of formaldehyde,[11] it is also important to determine that the loading and/or excitation of BCECF have no toxic effects on respiratory rhythmogenesis or central respiratory chemoreception.

Here we describe methods that will validate the use of optical recordings of pHi from neurons in the spontaneously respiratory rhythmic in the *in vitro* tadpole brainstem preparation. We have chosen to develop the optical recording of pHi in the *in vitro* tadpole brainstem preparation because it has been documented that this preparation is well-oxygenated and,[14] more importantly, that the synaptic connectivity of respiratory central pattern generators, central chemoreceptors and respiratory motor neurons remains intact in this preparation.[15–20] The ability to monitor whole nerve respiratory activity and changes in pHi simultaneously provides a powerful tool to dissect the mechanistic connections among the neural elements involved in respiratory responses to CO_2. It is essential to demonstrate that the use of ratiometric fluorescent dyes such as BCECF do not disrupt or modulate respiratory rhythmogenesis or central respiratory chemoreception. While many other preparations are useful in describing the neural control of respiration, the *in vitro* tadpole brainstem preparation presents several advantages over mammalian preparations with respect to developing the technique to perform simultaneous optical recordings of pHi and respiratory motor output. One very attractive feature of the *in vitro* tadpole brainstem preparation is its retention of synaptic connectivity. Disruption in synaptic connectivity occurs in all slice preparations because of destruction of dendritic arbors. The tadpole brainstem contains distinct chemosensitive sites and like mammalian preparations, contains chemosensitive projections on the medullary surface. The medullary slice and *en bloc* neonatal rodent preparations appear to contain all essential elements necessary for spontaneous respiratory rhythmogenesis. The reduced nature of the synaptic connections in these preparations, however, limits investigators' ability to examine the effects of more extensive neuronal connections on respiratory rhythmogenesis. For example, the absence of synaptic connectivity from many of the widely distributed central respiratory medullary chemoreceptors in the neonatal rodent slice preparation makes it difficult to investigate the effects of these extremely important sensors on the properties of respiratory rhythmic neurons. In contrast, the *in vitro* tadpole preparation facilitates such study. It retains all medullary chemoreceptive inputs, as evidenced by a robust fictive ventilatory response to central respiratory chemoreceptor stimulation. Other attractive features of the *in vitro* tadpole preparation are that it is studied at physiological temperatures (20–24°C), it does not require elevated extracellular potassium concentrations to enhance neural activity, and it remains well oxygenated. Finally, the inaccessibility of mammalian fetal life makes developmental studies in mammals difficult; the *in vitro* tadpole brainstem preparation is readily accessible throughout development and therefore facilitates developmental studies.

METHODS - *IN VITRO* BRAINSTEM PREPARATION

Animals

Experiments were performed on *Rana catesbeiana* tadpoles of various developmental stages of either sex obtained from a commercial supplier (Sullivan, TN, USA). All tadpoles were housed in aquaria and were maintained at 24–26°C with a 12h:12h light:dark photoperiod. Tadpoles were fed on a daily basis (Fish Flakes, Wardly, AZ, USA) as needed so that food was not a limiting resource. Animal care and protocols were approved by the University of Texas at San Antonio Institutional Animal Care and Use Committee.

Dissection

Tadpoles were anesthetized in 3-aminobenzoic acid ethyl ester (MS 222; Sigma-Aldrich, AZ, USA) dissolved in dechlorinated water (1:10,000) until unresponsive to tail pinch and weighed. The brainstem was dissected as previously described.[11] Throughout the dissection, the brainstem was continuously superfused with artificial cerebrospinal fluid (aCSF) bubbled with O_2 and CO_2 to attain a pH = 7.8. The composition of the aCSF was as follows (in mM): NaCl 104; KCl 4.0; $MgCl_2$ 1.4; D-glucose 10; $NaHCO_3$ 25; and $CaCl_2$ 2.4. Following dissection, the brainstem was incubated in oxygenated aCSF containing BCECF-AM (Molecular Probes, OR, USA). The aCSF was bubbled with O_2 and CO_2 to attain a pH = 7.8. The brainstem was removed as described above and transferred to a superfusion recording chamber.[14]

Whole Nerve Recordings

Neural recordings of fictive gill and lung ventilation were obtained from the roots of cranial nerve (CN) 7 using glass suction electrodes.[14–17] Efferent neural activity was amplified (10,000X; AM Systems, 1700), filtered (10 Hz to 500 Hz), simultaneously averaged with a moving time averager (CWE, MA-821, PA, USA; time constant = 200 msec.), digitized and recorded on a Pentium 4 PC (Datapac 2000 software, Run Technologies, CA, USA).

Pre-dye Neural Recordings

In order to investigate the central CO_2 chemoreceptive response before BCECF was loaded, the tadpole brainstem (n=10) was randomly exposed to normocapnic (pH=7.8) and hypercapnic conditions (pH 7.4 and 7.2). The superfusate was equilibrated in an external tonometer with gas having P_{CO2} values ranging from approximately 13–18 mm Hg, 31–38 mm Hg, and 55–64 mm Hg (balance O_2) to achieve pH values of 7.8, 7.4, and 7.2, respectively. Within two minutes after a stable pH was achieved in the tonometer, the pH in the recording chamber was equal to the tonometer pH ± .01 pH units.[14,16] After each 10 minute recording of spontaneous respiratory motor output, the P_{CO2} was adjusted to the new target pH followed by a five minute period to permit equilibration of the recording chamber pH and stabilization of the brainstem response to the new superfusate pH. After the last recording, CN 7 was detached from the suction electrodes and the brainstem was removed from the recording chamber to be loaded with BCECF dye.

Post-dye Neural Recordings

Immediately following the 30 minute loading period using BCECF-AM in aCSF, the brainstem was placed in the recording chamber where the suction electrode was re-attached to CN 7 for subsequent recordings of respiratory motor output. The post-dye protocol began after the brainstem had been superfused with aCSF without added BCECF-AM in the recording chamber for at least 60 minutes (as previously described for pre-dye recordings). Nerve activity was recorded from CN VII following the same normocapnic and hypercapnic protocol used for the pre-dye recordings.

We did not expose the dye loaded brainstem to either the 440 or 495 nm wavelengths of light because we wanted to document the effects of the presence of BCECF within brainstem neurons on respiratory motor output. A subset of experiments were performed in which the brainstem was loaded with BCECF as described above and whole nerve activity was recorded while exposing the brainstem to the paired 440 and 495 nm wavelengths of light every 30 seconds for 2 hours. We observed no significant changes in respiratory motor output or chemosensitivity while exposing the BCECF loaded brainstem to the excitatory wavelengths of light.

Dye Loading

BCECF-AM stock 0.004 M (Molecular probes, OR, USA) was made by dissolving 1 mg BCECF-AM in dimethylsulfoxide (DMSO) (20 µL). We incubated the *in vitro* tadpole brainstem (n=20) in 10 mL of 20, 40, or 60 µM solutions, by diluting the BCECF stock solution in the aCSF. The aCSF was bubbled with O_2 and CO_2 such that the pH was 7.8. The tadpole brainstem was incubated in BCECF, prepared as described above, for 30 minutes in the dark. Following dye incubation, the brainstem was washed for 60 minutes in oxygenated aCSF with CO_2 such that the pH was 7.8

Imaging of BCECF-loaded Neurons

The superfusion chamber containing the brainstem was placed under an upright microscope (ECLIPSE E600FN Nikon, NY, USA) mounted with a charge-coupled device (CCD) camera (MicroMAX, Roper Scientific, NJ, USA) connected to a Pentium computer (Hewlett Packard Company, CA, USA). Neurons were excited for 200–400 msec with light from a 175-W xenon arc lamp (Sutter Instrument, CA, USA) that was filtered to 495 or 440 nm using a high speed filter changer (Lambda DG-4, Sutter Instrument, CA, USA). Emitted light passed through a dichroic mirror with a high pass cut off of 515 nm and a 535 ± 25 nm emission filter (Chroma Technology, VT, USA). To determine the percentage of cell loading, the brainstem was sectioned after each experiment into 500 µm slices for subsequent differential interference contrast (DIC) microscopy. The same image was then exposed to the 440 nm excitation wavelength, making visible all cell bodies that were loaded with BCECF. The DIC image identified all cells and the image obtained after the 440 nm excitation identified the BCECF-loaded cells. The two images were merged to obtain the fraction of total cells in a slice loaded by BCECF. Data were collected with an image acquisition software program (MetaMorph/MetaFluor, Universal Imaging Corporation, PA, USA) for offline analysis.

RESULTS

Dye Loading

An overlay of the DIC and BCECF fluorescence is shown in Figure 1. Differential interference contrast (DIC) and fluorescence microscopy were performed on sequential 500 µm slices of the brainstem following incubation in 20, 40, or 60 µM concentrations of BCECF. Analysis software was used for the detection of dye within cells in these brainstem sections. The percentage of cells loaded with the 20 µM BCECF (33.2% ± 10.7%) was significantly lower than the 40 µM (83.4% ± 13.6%) and 60 µM (90.1% ± 4.1%) concentrations of BCECF. There was no significant difference in the percentage of cells loaded with dye between the 40 and 60 µM concentrations of BCECF.

Effects of BCECF on Respiratory Rhythmogenesis and Central Respiratory Chemoreception

Ester hydrolysis leads to release of formaldehyde. To ensure that BCECF concentrations used do not have toxic effects on ventral medullary

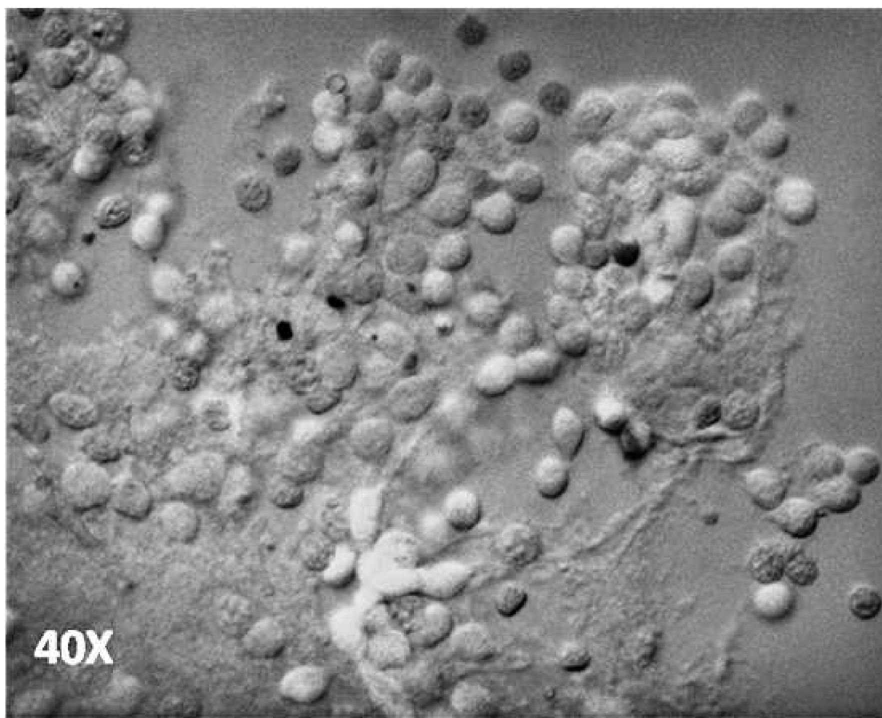

Fig 1. Overlay image of DIC (gray) and 440 nm excitation (light gray). Images were used to calculate BCECF percent loading

cells that would affect respiratory chemosensitivity or rhythm, whole nerve respiratory responses were monitored during control and hypercapnic solutions.

Respiratory Burst Activities and Respiratory Response to CO_2

Prior to incubation in BCECF, all *in vitro* tadpole brainstem preparations exhibited spontaneous gill and lung burst activities. Although the criteria for gill and lung bursts have been described,[3,14–20] gill bursts occur at a relatively low amplitude and high frequency whereas lung burst occur at relatively low frequency and high amplitude. The mean gill burst frequency during normocapnia (bath pH 7.8) was 39.2 ± 6.7 min^{-1}. Gill burst frequency was significantly reduced to 25.6 ± 3.9 min^{-1} with hypercapnia (bath pH 7.2). Lung burst frequency during normocapnia was 1.2 ± 0.3 min^{-1}. Lung burst frequency significantly increased to 8.4 ± 1.7 min^{-1} with hypercapnia (bath pH 7.2).

Gill and lung burst activities were retained in all brainstem preparations following incubation with 40 μM BCECF, as illustrated in Figure 2. In addition, following BCECF loading the gill burst frequency at normocapnia (34.5 ± 3.9 min^{-1}) was not significantly different from the gill burst frequency prior to BCECF incubation (39.2 ± 6.7 min^{-1}). Following dye loading with BCECF, gill burst frequency during hypercapnia (pH 7.2; 20.9 ± 1.8 min^{-1}) was significantly less than gill burst frequency during normocapnia (pH 7.8; 34.5 ± 3.9 min^{-1}). Lung burst frequency during normocapnia following exposure to BCECF (1.3 ± 1.4 min^{-1}) was not significantly different from the lung burst frequency during normocapnia prior to BCECF exposure (1.2 ± 0.3 min^{-1}). The significant increase in lung burst frequency during hypercapnia observed prior to BCECF exposure was conserved following BCECF exposure, as lung burst frequency significantly increased from 1.2 ± 0.3 min^{-1} during normocapnia to 6.7 ± 1.8 min^{-1} with hypercapnia. The gill and lung burst activities during control (bath pH = 7.8) and hypercapnia (bath pH = 7.2) before and after BCECF loading are illustrated in Figure 3.

Our ability to record gill and lung burst activities and observe a neuroventilatory response to hypercapnia also indicates that DMSO, the vehicle for the BCECF, did not produce detrimental effects on respiratory rhythmogenesis or central respiratory chemoreception. Our data also indicate that DMSO did not modulate respiration, as there were no significant differences in gill or lung frequencies at each bath pH before or after BCECF loading. We also exposed the BCECF loaded brainstem to continuous excitation (440 and 495 nm) every 30 seconds for two hours while recording gill and lung activities from CN 7. There were no significant changes in respiratory motor output or chemosensitivity in response to exposing the BCECF loaded brainstem to the excitatory wavelengths of light.

DISCUSSION

The use of fluorescence pH indicators to study the changes in pHi, and more importantly the regulation of pHi, can provide valuable insight into the function of central respiratory chemoreceptors. We performed experiments to validate the use of the pH sensitive dye, BCECF, in the *in vitro* tadpole brainstem preparation. The *in vitro* tadpole brainstem preparation offers a unique opportunity to investigate the neural development of respiratory rhythmogenesis and central respiratory chemoreception, as the synaptic connections of central respiratory pattern generators, central respiratory chemoreceptors and respiratory motor neurons remain intact

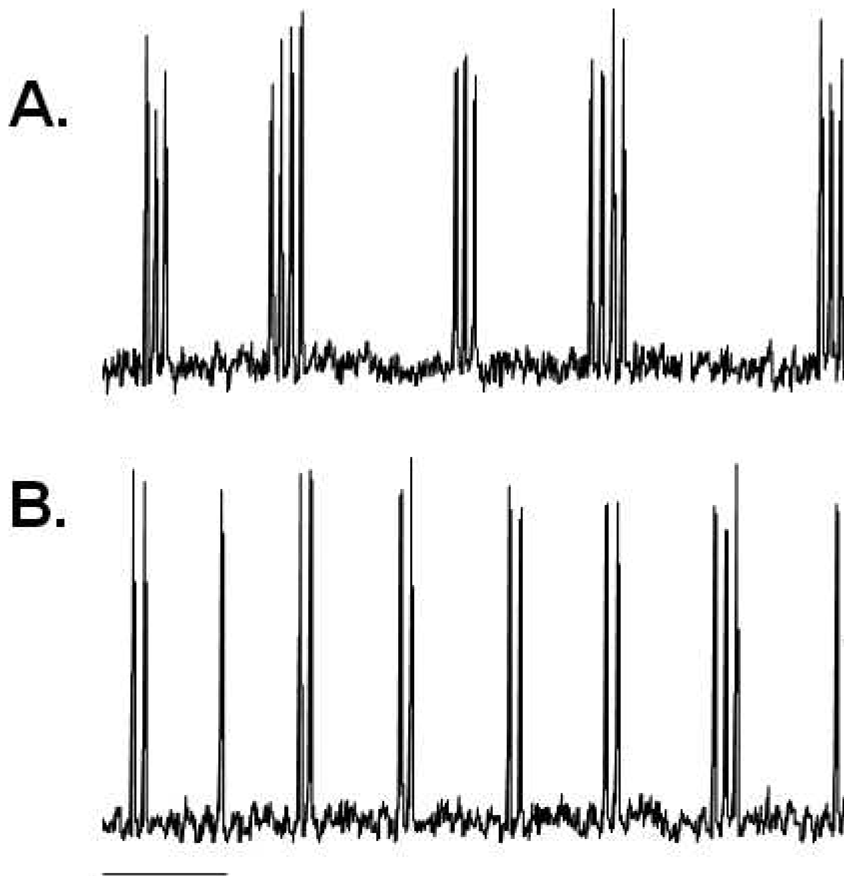

Fig 2. Gill and lung burst activities recorded from cranial nerve VII prior to (A) and following 40 μM BCECF incubation (B)

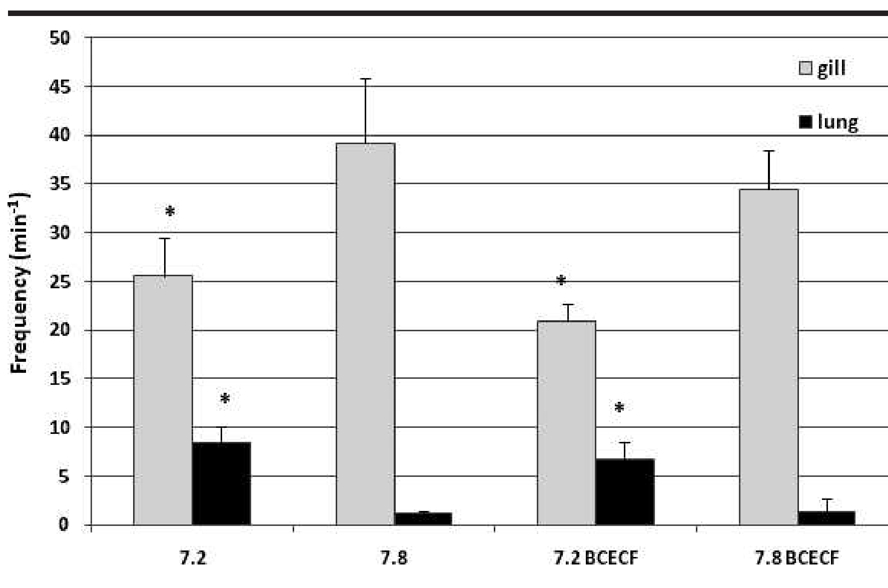

Fig 3. Gill and lung burst activities during control and hypercapnia before and after BCECF loading

in this preparation. We report four important findings regarding the valid use of BCECF in the tadpole brainstem. First, the percentage of cells loading the dye is 83%, which is similar to other experiments using mammalian preparations that yield 89% to 90% loading of BCECF.[21] Second, following the incubation period and washout, DIC and fluorescence microscopy indicate that BCECF undergoes cleavage of the –AM portion of the dye making it membrane impermeable, indicating that the tadpole brainstem neurons contain the appropriate intracellular esterases. Third, the whole nerve recording data demonstrate that there were no significant differences in the gill and lung burst frequencies prior to or after loading the cells with BCECF. Finally, we observed significant increases in lung burst frequency and decreases in gill burst frequency in response to central respiratory chemoreceptor stimulation before and after dye loading cells with BCECF. The presence of BCECF did not alter central respiratory chemoreceptor function, as the respiratory response to hypercapnia was retained following BCECF, and more importantly, the magnitude of the gill and lung respiratory responses to hypercapnia were not different before and after BCECF.

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